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3.	Full name, address and postcode of the or of each applicant *(underline all surnames)*	University of East Anglia Norwich, NR4 7TJ	
	Patents ADP number *(if you know it)*		
	If the applicant is a corporate body, give the country/state of its incorporation	United Kingdom	6778039001
4.	Title of the invention	Novel Therapeutic Target	
5.	Name of your agent *(if you have one)*	Keith W. Nash & Co.	
	"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*	90-92 Regent Street Cambridge CB2 1DP	
	Patents ADP number *(if you know it)*	1206001	

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Signature Keith W. Nash & Co. Date 31st January 2003

12. Name and daytime telephone number of person to contact in the United Kingdom — M. J. Lipscombe (01223) 355477

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Title: Novel Therapeutic Target

Field of the Invention

The present invention relates to methods of downregulating responses, *in vivo* or *in vitro*, to hormones of the TGFβ superfamily, to pharmaceutical compositions for such purposes, a method of making a pharmaceutical composition, and to use of certain substances to downregulate responses to hormones of the TGFβ superfamily.

Background of the Invention

There is a group of secreted polypeptide hormones known collectively as the Transforming Growth Factor β (TGFβ) superfamily.

TGFβs control a broad range of normal biological activities including cell growth, bone development, cell migration, differentiation and apoptosis. However, aberrant TGFβ signalling is responsible for a number of developmental disorders, human cancers and other diseases (see, for example, Massague *et al.*, 2000 Cell 103, 295-309).

Recently the signal transduction pathways, by which cells detect and respond to the presence of TGFβs have been at least partially elucidated, including the intracellular components which transduce TGFβ signals into the cell nucleus (reviewed by Moustakas *et al*, 2001 J. Cell Sci. 114, 4359-4369).

Genetic screens in Drosophila isolated a protein called MAD ('mothers-against-decapentaplegic') due to its involvement in the TGFβ signalling pathway known as Decapentaplegic (dpp). MAD-related proteins were subsequently identified in vertebrates and designated as Smad proteins. Smad proteins act downstream of the transmembrane

serine-threonine kinase receptors that mediate TGFβ signals (see Fig. 1). To date, 10 members of the Smad family have been described, and can be segregated into three functionally distinct sub-groups.

Upon activation, the TGFβ receptor complex induces phosphorylation of the receptor-regulated R-Smads (Smads 1, 2, 3, 5, 8). Receptors for TGFβ can activate Smad2, Smad3 and Smad8, and receptors for related factors (Bone morphogenic proteins, BMPs) activate Smad1 and Smad5. In the unstimulated state, R-Smads are maintained in an inactive conformation by internal interactions between conserved N-terminal Mad homology 1 (MH1) and C-terminal Mad homology 2 (MH2) domains. Phosphorylation of the C-terminal –Ser-Ser-X-Ser- motif in receptor-regulated Smads disrupts these auto-regulatory MH1-MH2 domain intramolecular interactions to facilitate Smad activation. In all cases, the phosphorylated R-Smads then associate with a common-mediator or co-Smad (Smad4). These heteromeric complexes are translocated to the nucleus, where they regulate gene transcription by either association with DNA-binding proteins or direct binding to promoter sequences in target genes.

Downregulation of TGFβ signalling is effected, in part, by a feedback mechanism that involves specific protein ubiquitination and proteasomal degradation of Smads. Ubiquitination plays a key role in a number of biological processes including signal transduction, cell cycle, and gene expression (Wilkinson, 2000 Cell Develop. Biol. 11, 141-148). Ubiquitination of proteins involves the concerted action of an E1 ubiquitin-activating enzyme, E2 ubiquitin conjugating enzymes, and E3 ubiquitin ligases that play a role in the specific recognition of target substrates. Recently, a new type of E3-type ubiquitin ligases, known as Smurfs, hve been shown to bind to Smads and have been implicated in their specific ubiquitination (see Fig. 2). Smurf1 can interact selectively with Smad1 (BMP pathway specific), and this mechanism appears to regulate the abundance of Smad1 in unstimulated cells since it is not affected by receptor activation. Smurf2 has been shown to interact with Smads 1, 2 and 3, however, only Smad2 becomes ubiquitinated and degraded by proteasomes. In this instance, Smad2 interaction with

Smurf2 is dependent upon receptor activation and the C-terminal phosphorylation of Smad2. In all cases, a small region in Smurfs known as a WW domain is responsible for the interaction with a –Pro-Pro-X-Tyr- sequence motif in Smads. Smad3 also undergoes ubiquitination by the SCF/Roc1 E3 ligase complex and subsequent degradation in the proteosome (Fukuchi *et al*, 2001 Mol. Biol. Cell 12, 1431-1443). In this instance, the ubiquitin ligase binds to a region in the C-terminal MH2 domain that is distant from the –Pro-Pro-X-Tyr- sequence motif in Smad3.

In view of the role that inappropriate TGFβ- induced responses play in a large number of diseases it would be useful to have an alternative means of downregulating TGFβ signalling. Such an alternative is provided by the present invention.

Summary of the Invention

The present inventors have identified a previously unknown interaction between Smad proteins and ubiquitin C-terminal hydrolases (UCHs). UCHs are enzymes which, as their name suggests, cleave ubiquitin. At least some UCHs are already well-characterised (see, for instance, Johnston *et al*, 1997 EMBO J. 16, 3787-3796; and Johnston *et al*, 1999 EMBO J. 18, 3877-3887). The association of Smad proteins with UCHs is thought likely by the inventors to result in stabilisation of the Smad, by inhibiting ubiquitin-mediated proteasomal degradation. Thus any method of preventing, inhibiting or reducing the association between Smads and UCHs should result in a downregulation of cellular responses to TGFβs and/or Bone morphogenic proteins (BMPs).

Accordingly, in a first aspect the invention provides a method of down-regulating cellular responses to TGFβs and/or BMPs, the method comprising the step of introducing into a cell a molecule which prevents, inhibits or reduces the association of Smad proteins with UCHs. The method may be performed on cells *in vitro* or *in vivo*.

In a second aspect the invention provides for use of a molecule which prevents, inhibits or reduces the association of a Smad protein with a UCH, for the downregulation of cellular responses to TGFβs and/or BMPs.

In a third aspect the invention provides for use of a molecule which prevents, inhibits or reduces the association of a Smad protein with a UCH in the preparation of a medicament to down-regulate cellular responses to TGFβs and/or BMPs.

In a fourth aspect the invention provides a pharmaceutical composition for down-regulating cellular responses to TGFβs and/or BMPs, the composition comprising a molecule which prevents, inhibits or reduces the association of a Smad protein with a UCH, in admixture with a physiologically acceptable carrier, excipient or diluent.

In a fifth aspect the invention provides a method of screening a test substance for the ability to prevent, inhibit or reduce the association of a Smad protein with a UCH, the method comprising the step of contacting the test substance with a Smad protein and/or a UCH and determining, qualitatively or quantitatively, the amount of association of the Smad protein with the UCH when these are contacted. The determination may be made in absolute or relative terms. Conveniently one or more of the test substance, Smad protein and UCH may be labelled with a readily detectable label such as a radio label, fluorophore, chromophore, antibody or the like. In a particular embodiment the method of screening may make use of, for example, a cell or cell extract. Test substances identified by the screening method may be of potential usefulness as drugs to downregulate cellular responses to TGFβs and/or BMPs. The screening method of the invention may comprise one, two or all of the following: ELISA; co-immunoprecipitation; Western blotting.

More especially, the invention applies in particular to the down-regulation of responses to TGFβs rather than to BMPs, since the association of UCHs is probably strongest with Smad3, which protein is involved in transduction of TGFβ signals but not transduction of BMP signals. Thus the invention especially relates to methods, uses and compositions for preventing, inhibiting or reducing the association between Smad3 and UCHs.

In particular, the association of Smad3 is believed to be strongest with UCH-L5 (a mouse UCH), or with the corresponding human homologue UCH37. Thus the invention in

preferred embodiments relates to a method of or composition for preventing, inhibiting or reducing the association between Smad3 and UCH-L5 or UCH37.

The present invention contemplates the use of any molecule which can have the desired effect, which may particularly be achieved, for instance, by:

(i) using a molecule which is a structural analogue of the UCH-binding site on Smad proteins which can therefore reversibly or irreversibly compete with a UCH for binding to Smads;

(ii) using a molecule which is a structural analogue of the Smad-binding site on UCHs (localised to at least the N terminal 195 amino acid residues of, for example, UCH-L5), which can therefore reversibly or irreversibly compete with Smad proteins for binding to UCHs;

(iii) using of a molecule which (preferably specifically) reduces the effective intracellular concentration of UCH e.g. by promoting degradation of UCHs. An example of such a molecule is ubiquitin aldehyde (or Ubal). Ubiquitin aldehyde is a ubiquitin derivative in which the C terminal carboxylate group is replaced by an aldehyde, and is a potent inhibitor of UCHs. (See, for example, Johnston *et al*, 1999 EMBO J. 18, 3877-3887; and Hu *et al*, 2002 Cell 111, 1041-1054.)

Within molecules of category (i), the inventors have been able to establish that a portion of Smad3 present within residues 144-240 are essential for UCH-L5 or UCH37 to bind to Smad3. The sequence of human Smad3 has been published in Nature (vol. 383, 1996 p168-172) and is available from Genbank (accession no. U68019). The amino acid sequence of the human Smad3 protein is shown, using single letter code, in Figure 7. Residues 144-240 are shown italicised and underlined.

Thus, in some embodiments, the method of the invention may comprise introduction into the cell (within which the response to TGFβ is to be downregulated) of a molecule which comprises a peptide having at least 60% sequence identity, preferably at least 70%, more

preferably at least 80%, and most preferably at least 90% sequence identity with a contiguous portion of Smad3 present within amino acid residues 144-240 of Smad3. Typically the molecule introduced into the cell will comprise a peptide of at least 8 amino acid residues having the desired level of sequence identity with the corresponding contiguous portion of Smad3, preferably at least 10 amino acid residues, more preferably at least 12 amino acid residues and most preferably 15 or more amino acid residues. The peptide will preferably comprise no more than 80 amino acid residues, more preferably no more than 60 amino acid residues, and most preferably no more than 40 amino acid residues.

The molecule may comprise modified or non-naturally occurring amino acid residues and/or non-peptide moieties in order to optimise the pharmacokinetic characteristics (e.g. increase stability [e.g. resistance to protease-mediated degradation]; reduce toxicity, and/or increase bioavailability). For example, the molecule may comprise a lipid or other hydrophobic moiety in order to improve transport across the cell membrane. Alternatively the molecule could be incorporated into or within a particulate vector, such as a liposome. Numerous suitable liposomes are known to those skilled in the art.

Where the molecule of use in the method consists of a peptide or small protein, it may be preferable to introduce into the cell a nucleotide sequence (typically a DNA sequence) which directs the expression in the cell of the effector peptide or protein. Nucleotide sequences can be introduced into cells *in vitro* or *in vivo* by a number of well known techniques including transfection, transduction by viral vectors (e.g. vaccinia virus and modified vaccinia virus ankara [MVA], adenovirus and the like), and by use of "gene guns" and so on.

Molecules which specifically reduce the effective intracellular concentration of UCHs may include UCH-specific proteases or molecules which interfere with the expression of UCHs. In this respect UCH-specific ribozymes or RNAi approaches may usefully be employed.

The pharmaceutical composition of the invention may be administered to a human or animal (preferably mammalian) subject by any convenient means: orally; by injection – intravenously, subcutaneously or, intramuscularly; intranasally; topically; rectally and so on.

Typically the pharmaceutical composition may comprise the active agent at a concentration in the range 0.01mg/gm to 100mgs/gm, more preferably in the range 0.1mg/gm to 10mgs/gm. A suitable dose can readily be ascertained for a particular subject by trial-and-error – a minimal dose may be administered for say 24-48 hrs, and the dose gradually increased (typically in a stepwise manner) until a therapeutic benefit or an adverse reaction is observed. A therapeutic benefit may be defined as any improvement in a subject's clinical condition which is recognisable by a suitably-qualified health professional and/or may readily be quantified relative to any absolute or relative index (e.g. size of a tumour).

The invention will now be further described by way of illustrative example and by reference to the accompanying drawings, in which:

Figure 1 is a schematic representation of the TGFβ and BMP signalling transduction pathways in a eukaryotic cell:

Figure 2 is a schematic representation of the ubiquitin-mediated proteasomal degradation of Smad proteins;

Figures 3(i)-(iii) are pictures showing the results of various immuno-precipitation experiments;

Figure 4 is a schematic representation of Smad3 protein and various truncations thereof employed by the inventors, together with an indication of their relative strength of interaction with UCH37;

Figure 5 is a bar chart of change in luminescence (arbitary units) for cells transfected with different combinations of nucleic acid constructs;

Figure 6 is a schematic representation of the interaction of UCH37 with Smad3 and how this interaction protects the Smad protein from Ubiquitin-mediated proteosomal degradation; and

Figure 7 shows the amino acid sequence of human Smad3.

Examples

The inventors used a yeast two-hybrid approach to identify proteins that interact with Smad3 and potentially regulate the TGFβ signalling pathway. A mouse brain cDNA library was screened and the positive clones were identified by sequencing and subsequent BLAST DNA database searches. Using this approach, the inventors found a Smad-interacting protein which was identified as a ubiquitin C-terminal hydrolase known as UCH-L5 (Genbank No. NM 019562 or AF148447) in mouse or UCH37 in humans (Genbank No. AF147717).

The yeast two-hybrid materials are commercially available from Clontech Laboratories Inc. (1020 East Meadow Circle, Palo Alto, CA 94303, USA). The technique is described in detail in the "MATCHMAKER GAL4 Two-Hybrid System 3 and Libraries User Manual" (PT3247-1 [PR94575]) published by Clontech, June 1999 (see also MATCHMAKER Two-Hybrid System 3, Jan. 1999 *CLONTECH*niques XIV(1):12-14).

In their screen, the inventors used sequences comprising residues 1-240 of Smad3 ($Smad3_{1-240}$) as bait in the Clontech yeast two-hybrid system. Interacting proteins were then investigated by co-expression and co-immunoprecipitation experiements using epitope-tagged proteins.

In these experiments, a FLAG^{RTM}-tagged full length UCH-L5 or a truncation lacking a C-terminal extension downstream of the N-terminal enzymatic domain (UCH-L5ΔC lacking residues Trp_{196}-Lys_{329}) was co-expressed with Haemagglutinin (HA)-tagged Smad proteins. Expression of these proteins was confirmed by western blot and interactions were identified by anti-HA western blot probing of anti-FLAG immunoprecipitates. The experimental techniques used are routine for those skilled in the art and are described, for example, by Sambrook *et al.* (Molecular Cloning. A Laborarory Manual. 2nd edition. Coldspring Harbor Press, Coldspring Harbor. USA) and by Wicks *et al.*, (2000 Mol. Cell. Biol. 20, 8103-8111).

By way of explanation, the FLAG^{RTM} tag (FLAG is a registered trade mark of Sigma-Aldrich Biotechnology LP) is a short peptide tag (amino acid sequence DYKDDDDK) which is incorporated into proteins expressed using the commercially available pFLAG^{RTM} expression construct (pFLAG is a registered trade mark of Sigma-Aldrich Biotechnology LP). Monoclonal antibodies are available which are specific for the FLAG^{RTM} peptide and so can be used to detect FLAG^{RTM}-labelled proteins. The FLAG^{RTM} system is further detailed and described in EP 0150126 and EP 0335899.

C-terminally FLAG^{RTM}-tagged UCH-L5 or UCH-L5ΔC were co-expressed in human embryonic kidney (HEK) – 293 cells with N-terminally HA-tagged Smad proteins. The results of the immunoprecipitation experiments are presented in Figures 3(i)-(iii).

Referring to Figure 3, HEK-293 cells were transfected with 20μg of DNA construct directing the expression of UCH-L5 FLAG or UCH-L5ΔC FLAG and 20μg of DNA construct expressing one of: HA-Smad 3_{1-385}; HA-Smad3_{1-240}; or HA-Smad3_{1-44}. Lysates of the cells were prepared by a standard detergent lysis method, using 1% Triton X-100 and then immunoprecipitated with an anti-FLAG monoclonal The precipitated proteins were then subjected to SDS-PAGE. The results are shown in Figure 3(iii).

For comparison, a blot of the same samples was then probed with a haemagglutinin-specific 1st antibody. Clear bands were detected corresponding to Smad 3_{1-385} or $Smad3_{1-240}$: these proteins had therefore been co-immunoprecipitated with UCH-L5 or UCH-L5 ΔC, demonstrating that the UCH-L5 or L5ΔC proteins must have been complexed with the $Smad3_{1-385}$ or $Smad3_{1-240}$ proteins. In contrast, no band could be detected at the position corresponding to $Smad3_{1-144}$, indicating that this protein was not bound by UCH-L5 or L5 ΔC. From this, the inventors deduced that the binding site for UCH-L5 on Smad3 must encompass at least a portion of the Smad3 protein present between residues 144 and 240.

Figure 3(ii) shows the results of the control experiment in which whole cell lysates of HEK-293 cells were run on a gel, blotted, and probed with an anti-haemagglutinin 1st antibody. These confirmed that the small $Smad3_{1-144}$ truncated protein was being expressed by the cells and therefore the failure to detect the protein in the immunoprecipitated material must have been due to its lack of binding to the UCH-L5 FLAG or UCH-L5ΔC FLAG proteins. The results in Figure 3 also demonstrate that there is no significant difference between the results for UCH-L5 or for the C-terminally truncated version UCH-L5ΔC.

Example 2

In the light of the foregoing results the inventors decided to investigate further the interaction between Smad3 and UCH-L5. In particular the inventors wished to discover if over-expression of UCH-L5 could affect levels of Smad-dependent gene expression. To this end, they utilised a plasmid, SBE-luc (Labbé *et al*, 1998 Molec. Cell 2, 109-120) which expresses the luciferase reporter gene in a Smad-dependent manner, the luciferase-coding sequence being operably linked to a Smad binding element ("SBE").

HEK-293 cells were transfected with

(a) 5μg SBE-luc alone; or

(b) 5μg SBE-luc + 10μg UCH-L5 FLAG construct; or

(c) 5μg SBE-luc + 5μg TGF-β receptor construct; or

(d) 5μg SBE-luc + 10μg UCH-L5 FLAG construct and 5μg TGF-β receptor construct.

The TGF-β receptor construct directed the expression of the constitutively active type I receptor [TGF-βRI_{T204D}]).

The resulting level of luminescence, due to luciferase-activity, was assayed the luciferase reporter kit (Roche). The results are shown in Figure 5, which is a bar chart showing change in luminescence (arbitary units) for cells in groups (a)-(d).

The cells transfected with SBE-luc alone did not generate any luminescence. Co-expression of SBE-luc and UCH-L5 had no significant effect. Co-expression of SBE-luc and TGFβ receptor caused a modest increase in luminescence. Co-expression of SBE-luc simultaneously with both UCH-L5 and TGFβ receptor caused a significant (about 6 fold) further increase in luminescence.

In conclusion the inventors have identified a novel interaction between the Smad3 transcription factor and a ubiquitin C-terminal hydrolase and believe that this interaction could lead to stabilisation of the Smad3 protein and potentiation of TGFβ signalling by reversal of ubiquitin-mediated proteasomal degradation via ubiquitin ligase containing complexes such as SCF/Roc1. Targeting of a specific drug or peptide mimetic to the interaction domain between Smad3 (within residues 144-240) and UCH37 in humans could be useful to treat diseases or conditions in which there is over-activation of the TGFβ signalling pathway (Fig. 4). Examples of detrimental gain of TGFβ signalling can be found in fibrotic disease, wound healing/scarring, and eye diseases such as cataract. Inreases in TGFβ signalling are also thought to play a role in the late stages of cancer in which there is formation of new blood vessels (angiogenesis) that supports tumour growth, and metastatic migration of tumour cells.

CLAIMS

1. A method of down-regulating cellular responses to TGFβs and/or BMPs, the method comprising the step of introducing into a cell a molecule which prevents, inhibits or reduces the association of Smad proteins with UCHs.

2. Use of a molecule which prevents, inhibits or reduces the association of a Smad protein with a UCH, for the downregulation of cellular responses to TGFβs and/or BMPs.

3. Use of a molecule which prevents, inhibits or reduces the association of a Smad protein with a UCH in the preparation of a medicament to down-regulate cellular responses to TGFβs and/or BMPs.

4. A pharmaceutical composition for down-regulating cellular responses to TGFβs and/or BMPs, the composition comprising a molecule which prevents, inhibits or reduces the association of a Smad protein with a UCH, in admixture with a physiologically acceptable carrier, excipient or diluent.

5. A method of screening a test substance for the ability to prevent, inhibit or reduce the association of a Smad protein with a UCH, the method comprising the step of contacting the test substance with a Smad protein and/or a UCH and determining, qualitatively or quantitatively, the amount of association of the Smad protein with the UCH when these are contacted.

ABSTRACT

Title: Novel Therapeutic Target

Disclosed is a method of down-regulating cellular responses to TGFβs and/or BMPs, the method comprising the step of introducing into a cell a molecule which prevents, inhibits or reduces the association of Smad proteins with UCHs.

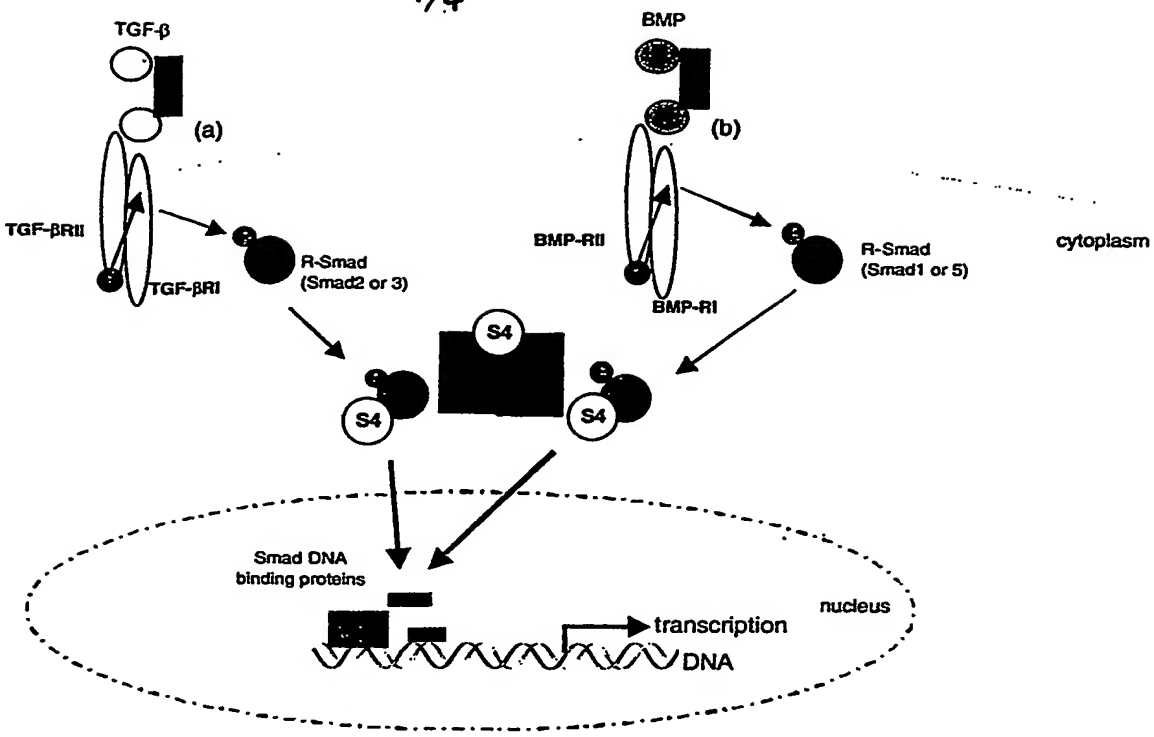


Figure 1. Diagrammatic representation of TGF-β receptor signalling to Smads
(a) Activated TGF-βRI associates with receptor-regulated Smads 2 or 3 (R-Smad). Subsequent R-Smad phosphorylation at C-terminal serines leads to hetero-oligomerisation with the common-mediator (Co-Smad), Smad4. The hetero-oligomeric complex is then translocated to the nucleus, where it binds directly, or in complex with other components, to DNA and affects transcription of specific genes. (b) Activated BMP-RI signals in a similar way to TGF-βRI. However it associates with and phosphorylates R-Smads 1 or 5 before hetero-oligomerisation with Smad4.

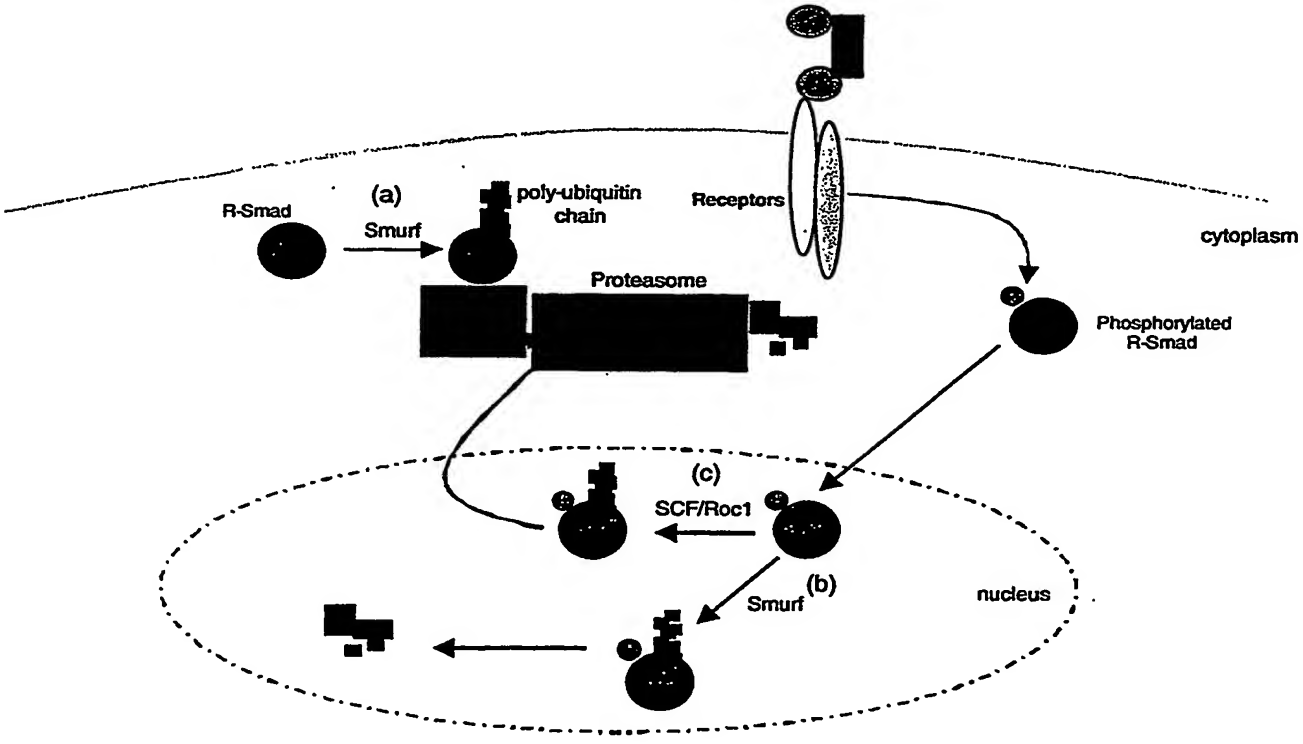


Figure 2. Targeted ubiquitination leads to Smad degradation (a) Cytoplasmic R-Smad ubiquitination and proteasomal degradation is mediated by Smurfs. (b) Nuclear activated R-Smads are degraded after Smurf-mediated ubiquitination. (c) Nuclear R-Smads are ubiquitinated by the action of the SCF/Roc1 E3 ligase complex, exported to the cytoplasm and undergo proteasomal degradation.

Fig 3

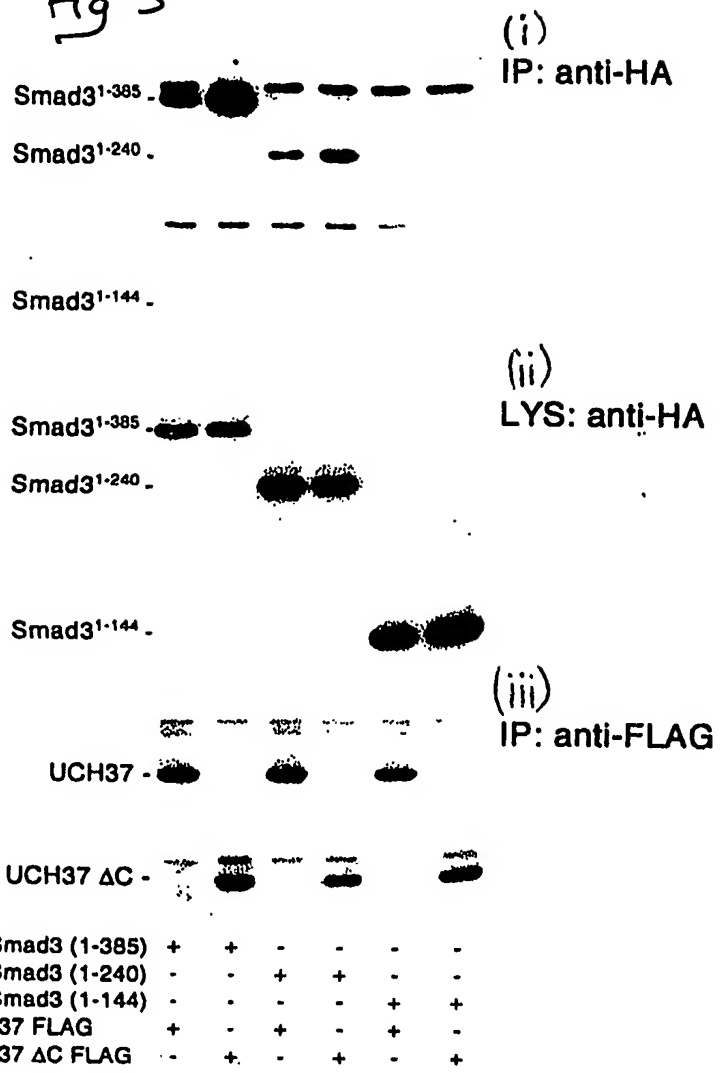
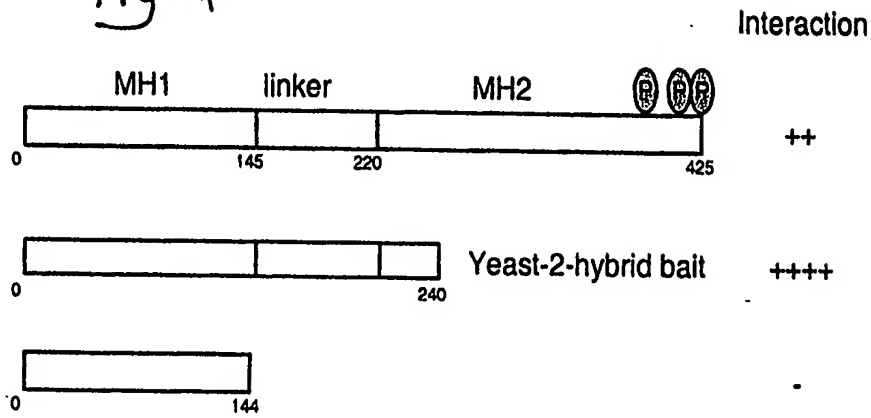


Fig 4



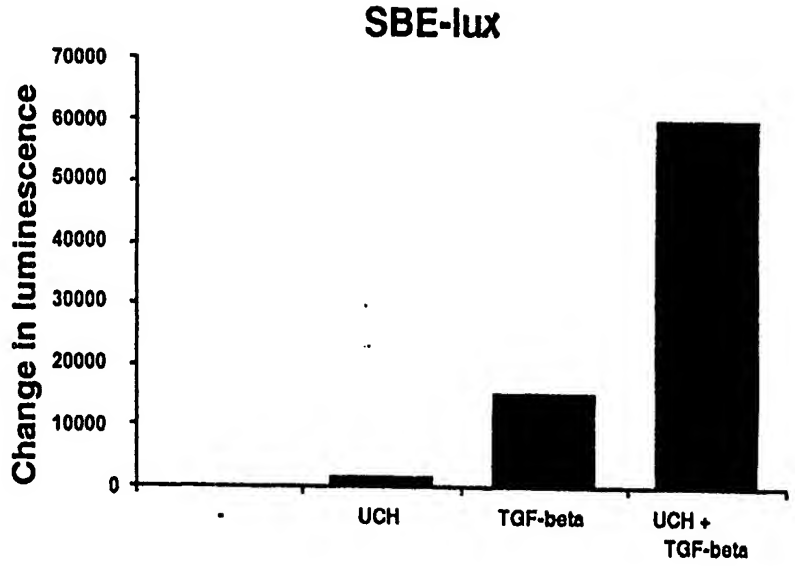


Fig 5

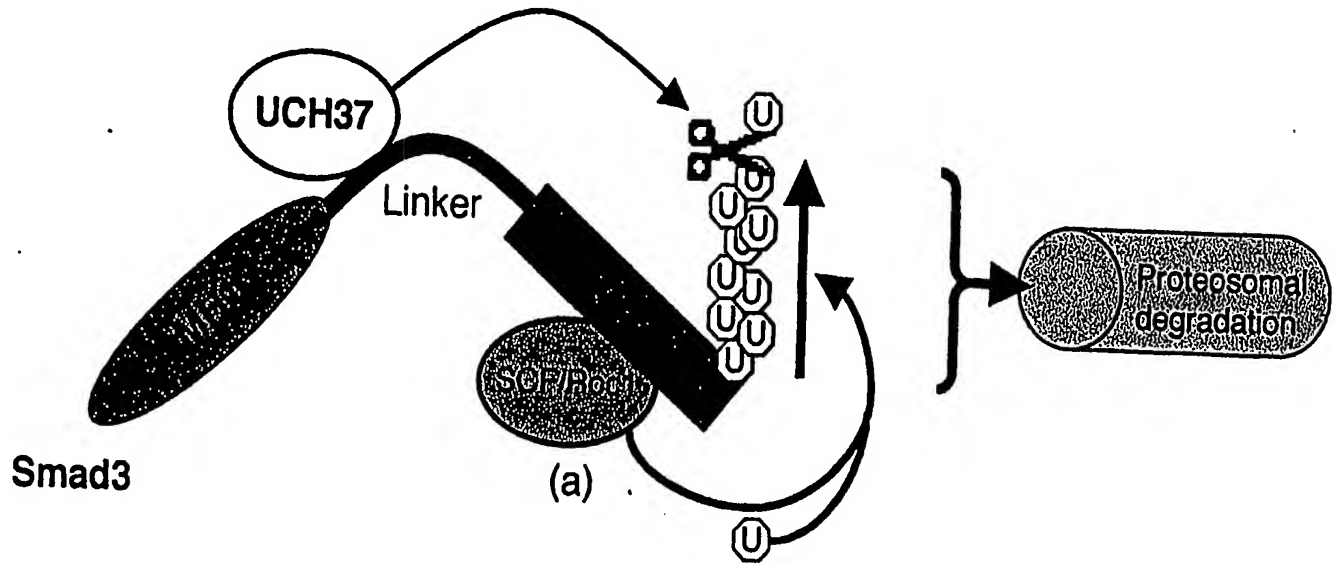


Figure 6 Removal of ubiquitin sub-units from the poly-ubiquitin chain on Smad3 by ubiquitin-C-hydrolase 37 (UCH37). (a) Nuclear R-Smads (eg. Smad3) are ubiquitinated by the action of the MH2 bound SCF/Roc1 E3 ligase complex, exported to the cytoplasm and undergo proteasomal degradation [Fukuchi *et al.* 1999]. (b) UCH37 which binds to Smad-3 in the region aa 144-240 facilitates the removal of ubiquitin and may prevent targeted proteasomal degradation of the Smad protein.

Figure 7 – amino acid sequence of human Smad3

MSSILPFTPPIVKRLLGWKKGEQNGQEEKWCEKAVKSLVKKLKKTGQLDELEKAITTQNVNTKCITIPRSLDGRLQVSHRKGLPHVIYCRLWRWPDLHSHHELRAME

LCEFAFNMKKDEVCVNPYHYQRVETPVLPPVLVPRHT*EIPAEFPPLDDYSHSIPENTNFPAGIEPQSNIPETPPPGYLSEDGETSDHQMNHSMDAGSPNLSPNPMSP*

*AHNNLDLQPVTYCEPAFWCSISYYEL*NQRVGETFHASQPSMTVDGFTDPSNSERFCLGLLSNVNRNAAVELTRRHIGRGVRLYYIGGEVFAECLSDSAIFVQSPNCN

QRYGWHPATVCKIPPGCNLKIFNNQEFAALLAQSVNQGFEAVYQLTRMCTIRMSFVKGWGAEYRRQTVTSTPCWIELHLNGPLQWLDKVLTQMGSPSIRCSSVS

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